Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2336–2339, March 1992
Medical Sciences

Direct effects of luteinizing hormone-releasing hormone agonists and antagonists on MCF-7 mammary cancer cells

(luteinizing hormone-releasing hormone analogues/receptors/cell growth)

TZVIA SEGAL-ABRAMSON*, HAVA KITROSER*, JOSEPH LEVY*, ANDREW V. SCHALLY†, AND YOAV SHARONI*‡

*Clinical Biochemistry Department, Faculty of Health Sciences, Soroka Medical Center, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel; and †Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center and Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70146

Contributed by Andrew V. Schally, December 9, 1991

ABSTRACT **The binding of luteinizing hormone-releasing hormone (LH-RH) analogues to the human mammary tumor cell line MCF-7 and their effect on the cell proliferation was studied to elucidate their direct action on estrogen-dependent mammary tumors. The growth rate of these cells was doubled by the addition of 1 nM estradiol to cells maintained in an estrogen-deficient medium. Although the basal growth rate was only slightly inhibited by the LH-RH antagonist [Ac-D-Nal(2)^1,D-Phe(pCl)^2,D-Pal(3)^3,D-Cit^6,D-Ala^{10}]LH-RH (SB-75), the estrogen-stimulated growth was completely abolished by the antagonist. In contrast, the LH-RH agonist buserelin stimulated cell growth in estrogen-deficient medium, whereas it had no effect in the presence of estrogen. ^{125}I-labeled buserelin was used for the measurement of LH-RH receptors on MCF-7 cells. A Scatchard plot analysis of buserelin-specific binding revealed a nonlinear plot, which suggested the presence of one high-affinity binding site with a K_d of 1.4 ± 1.0 nM and the remaining sites with low affinity (K_d = 1.3 ± 1.0 μM). The binding of ^{125}I-labeled buserelin was displaced equally well by unlabeled buserelin and by the LH-RH antagonist SB-75, suggesting that both analogues are bound to the same receptor. When parallel experiments were performed with ^{125}I-labeled SB-75, the binding was displaced by unlabeled SB-75 and other antagonists, but only partially displaced by unlabeled buserelin. The results suggest that in these mammary tumor cells there is a LH-RH antagonist binding site that is not recognizable by LH-RH agonists. This hypothesis was tested by measuring cell growth in the presence of both agonists and antagonists. It was found that SB-75 inhibited the stimulation of growth by buserelin, but buserelin did not prevent the inhibition by the antagonist of the estrogen-dependent growth. These results suggest that antagonists directly inhibit mammary tumor growth, not only by competing with LH-RH high-affinity receptors, but also by other mechanisms mediated by low-affinity antagonist binding sites.**

The growth of the human breast cancer cell line MCF-7, a well-accepted model for hormone-dependent breast cancer, is regulated by estrogens. The steroid action is probably mediated by the production of autocrine or paracrine growth factors, some of which have been studied extensively during the last years (1).

Since 1982 various studies have shown that synthetic analogues of the hypothalamic hormone luteinizing hormone-releasing hormone (LH-RH) have a therapeutic effect on sex steroid-dependent tumors such as carcinoma of the prostate (2) and breast (3, 4). The rationale for this treatment is that continuous administration of LH-RH agonists causes inhibition of gonadotropin release from the pituitary and thus reduces steroid production by the ovaries or testes.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Conflicting data exist pertaining to the possible direct effect of various LH-RH analogues on the growth of mammary tumor cells in culture. Miller *et al.* (5) were the first to propose that LH-RH and some of its agonists may inhibit the growth of mammary tumor MCF-7 cells in culture. However, other work has not been able to confirm these results (6). It was reported that, while LH-RH agonists have no significant effect on cell growth, some antagonists did inhibit thymidine incorporation in breast cancer cell lines (6). We have recently shown that a new generation of LH-RH antagonists inhibit cell proliferation in human estrogen-independent mammary cancer cells (MDA-MB-231), whereas buserelin {[D-Ser(tBu)^6,des-Gly^{10}-ethylamide]LH-RH}, a LH-RH agonist, has no significant effect (7).

Although chronic administration of potent agonists results in the inhibition of pituitary and gonadal function, it recently became clear that an antagonist may be clearly advantageous in the treatment of cancer as compared with the available superagonists. While repeated administration of the LH-RH agonist is required to inhibit pituitary and gonadal function, the same effect may be obtained by a single administration of the LH-RH antagonist (8). The inhibition of gonadotropin release by the LH-RH antagonist commences immediately after its administration, whereas agonists frequently cause a transient stimulation of pituitary and gonadal function, resulting in a temporary clinical "flare-up" of the disease (9).

The purpose of our present study was to check the direct effect of the LH-RH antagonist [Ac-D-Nal(2)^1,D-Phe(pCl)^2,D-Pal(3)^3,D-Cit^6,D-Ala^{10}]LH-RH (SB-75) on hormone-dependent mammary cancer cells in culture and to compare it with the effect of LH-RH agonists. The mechanism of the direct effect of these peptides on cancer cells was further studied by characterization of their binding to the tumor cells.

MATERIALS AND METHODS

Peptides. Buserelin was a gift from J. Sandow (Hoechst Pharmaceuticals); the antagonists SB-75 and [Ac-D-Nal(2)^1,D-Phe(pCl)^2,D-Pal(3)^3,D-Hci^6,D-Ala^{10}]LH-RH (SB-88) were synthesized and purified in the Veterans Administration Tulane University laboratory as reported (8).

Cell Culture. MCF-7 human mammary cancer cells were grown in 75-cm^2 flasks in Dulbecco's modified Eagle's medium (Biological Industries, Beth Haemek, Israel) containing

Abbreviations: LH-RH, luteinizing hormone-releasing hormone; SB-88, [Ac-D-Nal(2)^1,D-Phe(pCl)^2,D-Pal(3)^3,D-Hci^6,D-Ala^{10}]LH-RH; SB-75, [Ac-D-Nal(2)^1,D-Phe(pCl)^2,D-Pal(3)^3,D-Cit^6,D-Ala^{10}]LH-RH; Cit, citrulline; Hci, homocitrulline; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; FCS/DCC, charcoal stripped fetal calf serum; Nal(2), 3-(2-naphthyl)alanine; Phe(pCl), 4-chlorophenylalanine; Pal(3), 3-(3-pyridyl)alanine.

‡To whom reprint requests should be addressed.

Downloaded by guest on February 18, 2022

penicillin (100 units/ml), streptomycin (0.1 mg/ml), nystatin (12.5 units/ml), insulin (0.6 μg/ml), and 10% fetal calf serum.

Cell Growth. The cells were stripped of endogenous steroids according to Vignon *et al.* (10) by successive passages in medium without phenol red containing 10% then 3% charcoal-stripped fetal calf serum (FCS/DCC), before plating into 96-multiwell plates (12,000–15,000 cells per well) in a medium containing 3% FCS/DCC without insulin. One day later the medium was changed to 1% FCS/DCC and supplemented for various periods of time with hormones as indicated in the figure legends. Peptides were added every 2 days. After incubation, the number of cells was estimated by the cellular reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) by mitochondrial dehydrogenases of viable cells to a blue formazan product. When this product is dissolved in dimethyl sulfoxide, its absorbance, which is measured spectrophotometrically by an ELISA reader, is proportional to the number of cells (11). Every set of experiments was accompanied by a calibration curve from the same batch of cells. In preliminary experiments, the validity of the method was confirmed by cell counting as described previously (7).

Iodination of LH-RH Analogues. Buserelin and SB-75 were iodinated by the chloramine-T method, with modification according to Fekete *et al.* (12). An aliquot of the peptide (5 μg in 10 μl of 0.01 M acetic acid) was mixed with 40 μl of 0.5 M phosphate buffer (pH 7.4) and 1 mCi of $Na^{125}I$ (10 μl). Ten microliters of chloramine T (1 mg/ml) was added. After 20 sec, the reaction was terminated by adding 10 μl of sodium metabisulfide (1 mg/ml) and 120 μl of 0.5 M phosphate buffer (pH 7.4) plus 1 μl of 2.5 M KI. The purification of the labeled peptide was carried out by HPLC on a C_{18} reverse-phase column (LKB). The specific activity of the labeled peptides was about 600 cpm/fmol, determined by displacement curves performed with increasing concentrations of the labeled peptide alone.

Radioligand Binding Assays. Binding experiments were done essentially as described (13). Cells were grown in 150-cm^2 flasks, washed in phosphate-buffered saline (PBS; 8 mM sodium phosphate/1.5 mM potassium phosphate/2.7 mM KCl/137 mM NaCl, pH 7.4), scraped by a rubber policeman, and dispersed by repeated pipetting in a buffer containing 10 mM Tris·HCl, 0.1% crystalline bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, leupeptin (2 μg/ml), and 0.25 M sucrose (incubation buffer). Fifteen microliters of unlabeled peptides (various concentrations) solubilized in 0.005 M acetic acid and labeled peptide (100,000 cpm) were incubated with cell suspension in a total volume of 0.5 ml of incubation buffer for 90 min at 4°C. These experimental conditions were established as the most suitable in preliminary studies (data not shown). Incubation was terminated by the addition of 3 ml of PBS containing 0.1% bovine serum albumin and filtration on presoaked Whatman GF/C filters. The filters were washed three times with 3 ml of the same buffer and assayed in an LKB crystal multidetector γ system. Total binding was determined in the absence of unlabeled peptide, and nonspecific binding was determined in the presence of 10^{-5} M peptide, unless indicated otherwise. In regular experiments, the total binding for ^{125}I-labeled buserelin (^{125}I-buserelin) was between 7000 and 10,000 cpm, which represents, on the average, 6–8% of the total cpm present in the tube. For ^{125}I-labeled SB-75 (^{125}I-SB-75) the total binding was between 20,000 and 30,000 cpm, which represents, on the average, 20–30% of the total cpm present in the tube. The specific binding was 50–60% of the total binding. The K_d values and the number of receptors were analyzed as described by Munson and Rodbard (14) using the EBDA and LIGAND programs.

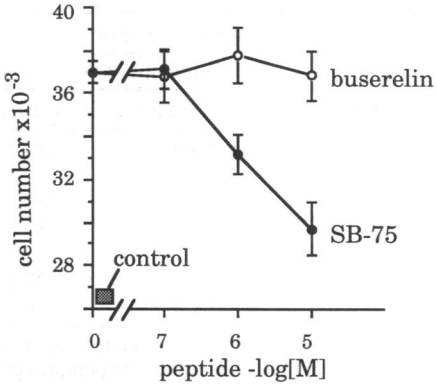

FIG. 1. Dose-dependent effect of LH-RH agonist (buserelin) and antagonist (SB-75) on estrogen-dependent MCF-7 cell growth. Estrogen-withdrawn cells were prepared as described in *Materials and Methods*. Cells were incubated in 96-multiwell plates for 4 days in the presence of 10^{-9} M estradiol plus the peptides at the indicated concentrations. The number of cells at the end of the incubation period was evaluated by the MTT method. The number of cells grown without estradiol (control) is shown for comparison. The results are the mean ± SEM of five different experiments, each done in 10 replicates.

RESULTS

Effect of LH-RH Agonists and Antagonists on MCF-7 Cell Growth. MCF-7 cells grow slowly in an estrogen-deficient medium. The average increase in cell number, during 4 days in culture, was 85% ± 4%, from 14,000 ± 800 to 26,000 ± 900. The growth rate of the MCF-7 cells was almost doubled, to 37,000 ± 700, by the addition of 10^{-9} M estradiol to cells grown in estrogen-deficient medium (Fig. 1). The estrogen stimulation was almost completely abolished in a dose-dependent manner by the LH-RH antagonist SB-75 (Fig. 1). Buserelin did not affect the cell growth induced by estradiol.

Buserelin stimulated the basal cell growth in the absence of estradiol (Fig. 2). The stimulatory effect of buserelin (10^{-5} M) was between 20% and 100% of the effect of estradiol (10^{-9} M). In 3 of 12 experiments, buserelin was not effective. [D-Trp^6]LH-RH, another potent agonist, had a smaller effect on cell growth (Table 1). Under this experimental condition (the absence of estrogen), the LH-RH antagonist SB-75 showed minor inhibition of MCF-7 cell growth.

^{125}I-Buserelin Binding to MCF-7 Cells. ^{125}I-Buserelin interacted with specific binding sites in MCF-7 mammary tumor cells. Bound ^{125}I-buserelin was equally displaced by the

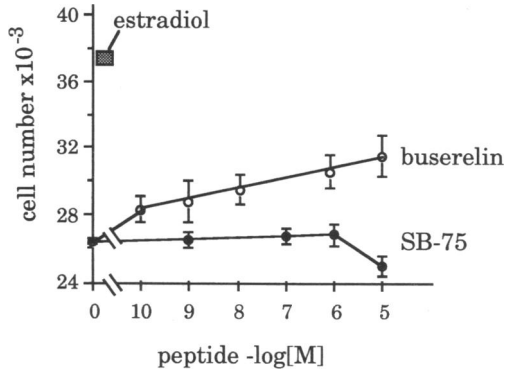

FIG. 2. Dose-dependent effect of LH-RH agonist (buserelin) and antagonist (SB-75) on MCF-7 cell growth. Estrogen-withdrawn cells were prepared as described in *Materials and Methods* and the legend to Fig. 1. Cells were grown for 4 days in the presence of the peptides at the indicated concentrations. The effect of 10^{-9} M estradiol on cell growth is shown for comparison. The results are the mean ± SEM of five different experiments, each done in 10 replicates.

Downloaded by guest on February 18, 2022

Table 1. Effect of various LH-RH agonists and antagonists on MCF-7 cell growth with and without estradiol

Addition	Cell number, % of control	
	− estradiol	+ estradiol
None	100 ± 2	188 ± 4
Agonists		
Buserelin	139 ± 6	187 ± 10
[D-Trp^6]LH-RH	124 ± 5	182 ± 5
Antagonists		
SB-75	87 ± 5	127 ± 10
SB-88	98 ± 4	118 ± 16

Cells were incubated in 96-multiwell plates for 4 days in the presence of either the peptides (10^{-5} M) or the peptides plus estradiol (10^{-9} M). The number of cells at the end of the incubation period was evaluated by the MTT method. The increase in cell number during the incubation without any addition was taken as 100%. The results are the mean ± SEM of five different experiments, each done in 10 replicates.

LH-RH agonists buserelin (Figs. 3 and 4) and [D-Trp^6]LH-RH (data not shown) and the antagonist SB-75 (Fig. 4). Unrelated peptides such as oxytocin and vasopressin did not affect buserelin binding (data not shown). Scatchard plot analysis of ^{125}I-buserelin binding was not linear, indicating the presence of at least two populations of binding sites (Fig. 3). Analysis by a two-site model showed high-affinity binding sites with a K_d of 1.4 ± 1 nM and a B_{max} of 36 ± 11 fmol/mg of cellular protein (24,000 receptors per cell) and low-affinity binding sites with a K_d of 1.3 ± 1 μM and a B_{max} of 42 ± 25 pmol/mg of cellular protein (28 × 10^6 receptors per cell).

Displacement of Labeled LH-RH Agonists and Antagonists from Mammary Tumor Cells by Unlabeled Peptides. Binding of ^{125}I-buserelin to mammary tumor cells (Fig. 4) was displaced equally well by unlabeled buserelin and by the LH-RH antagonist SB-75, suggesting that both analogues are bound to the same receptor. The binding of ^{125}I-SB-75 to these cells was displaced by unlabeled SB-75 and other antagonists, such as SB-88, but only partially by the agonist buserelin. These results suggest that there is a LH-RH antagonist binding site in mammary tumors that is not recognizable by LH-RH agonists. This hypothesis was tested by measuring the effect on cell growth of the simultaneous addition of agonist and antagonist.

Simultaneous Effect of LH-RH Agonists and Antagonists on MCF-7 Cell Growth. Buserelin stimulated cell growth in the absence of estrogen. This stimulation was completely abolished by SB-75 (Fig. 5). This is in full agreement with the complete displacement of ^{125}I-buserelin binding by the antagonist (Fig. 4). In the presence of estrogen, buserelin, when present at the same concentration as SB-75, or even at a 10-fold higher concentration, did not prevent its inhibition of cell growth. This result agrees well with the partial displacement of ^{125}I-SB-75 by buserelin.

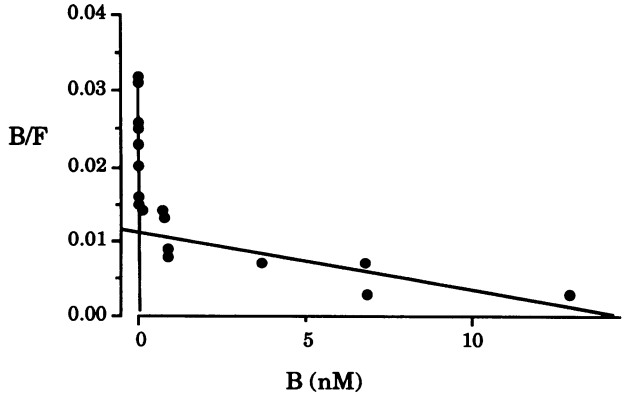

FIG. 3. Scatchard analysis of ^{125}I-buserelin binding to mammary tumor cells. Cells (3 × 10^6) were incubated with ^{125}I-buserelin (≈0.1 nM) and with increasing concentrations of unlabeled buserelin. The binding was performed as described in *Materials and Methods*. Analysis by a two-site model was performed with the LIGAND program (14). The graph is representative of three experiments, each done in duplicate, with comparable results.

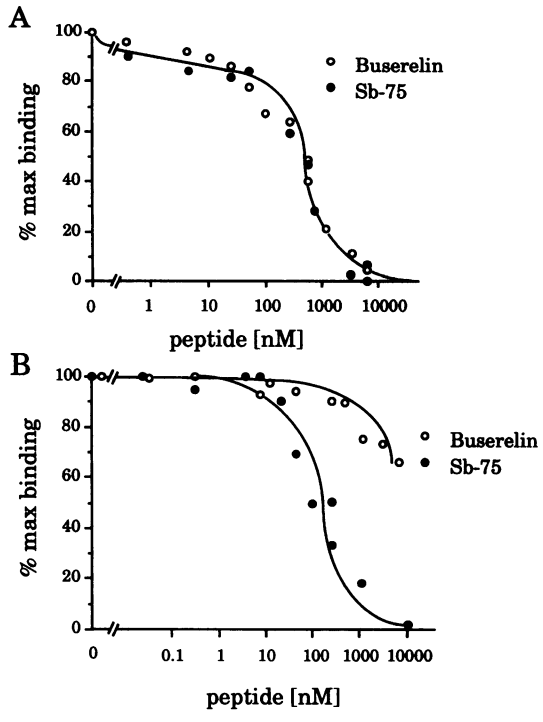

FIG. 4. Displacement of labeled LH-RH agonists and antagonists from mammary tumor cells by unlabeled peptides. For methodological details see the legend to Fig. 3. Mammary tumor cells were incubated with ^{125}I-buserelin (*A*) or ^{125}I-SB-75 (*B*) (≈0.1 nM each) and with increasing concentrations of unlabeled buserelin (○) or SB-75 (●). The graph represents three experiments, done in duplicate, with comparable results.

DISCUSSION

The main finding of this study is the direct effect of LH-RH analogues on the growth of human estrogen-dependent MCF-7 breast cancer cells. The antagonists SB-75 and SB-88 signif-

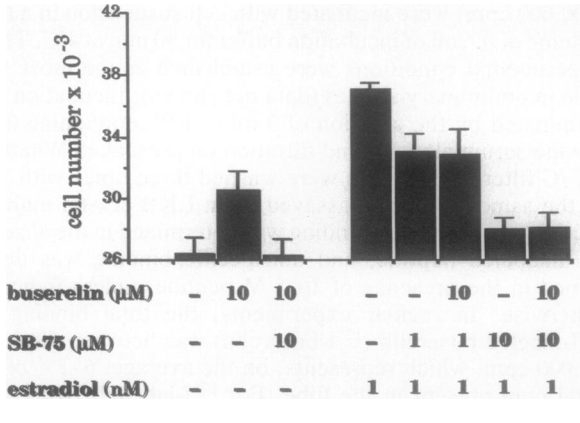

FIG. 5. Cell growth in the presence of simultaneous addition of LH-RH agonist (buserelin) and antagonist (SB-75). Estrogen-withdrawn cells were prepared as described in *Materials and Methods* and the legend to Fig. 1. The concentrations of added compounds are indicated in the figure. The results are the mean ± SEM of five different experiments, each done in 10 replicates.

Downloaded by guest on February 18, 2022

icantly inhibited estrogen-induced cell proliferation. These antagonists also partially inhibited the slow estrogen-independent growth of these cells. These results corroborate our recent studies with human estrogen-independent MDA-MB-231 breast cancer cells in culture (7). In that study the antagonists from the same series, [Ac-D-Nal(2)^1,D-Phe(pCl)^2,D-Trp^3,D-Hci^6,D-Ala^{10}]LH-RH (SB-29) and [Ac-D-Nal(2)^1,D-Phe(pCl)^2,D-Trp^3,D-Cit^6,D-Ala^{10}]LH-RH (SB-30), caused up to 60% inhibition of cell proliferation. This inhibition was dose dependent. Another older antagonist, ORG 30276 {[*N*-acetyl-D-Phe(pCl)^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]LH-RH}, also inhibited cell growth, but had a lesser effect. In these two breast cancer cell lines, buserelin, a LH-RH superagonist, did not inhibit cell growth.

Surprisingly, in MCF-7 cells, buserelin (and [D-Trp^6]LH-RH) stimulated proliferation of cells grown in the absence of estrogen. This was not seen in other studies with the same cell line (3, 5). The main dissimilarity is that in the previous studies the cells were grown in the presence of estrogens. Indeed, in our experiments, when performed in the presence of estradiol, buserelin did not stimulate cell growth. In addition to the known flare-up effect of LH-RH agonists mediated by the pituitary gonadal axis, we now show that growth stimulatory effects may be caused by the LH-RH agonists when acting directly on the tumor in the absence of estrogens. The practical conclusion of these results is that the treatment with LH-RH agonists might be risky for some breast cancer patients, because at the time when the agonist decreases estrogen blood level it may also directly stimulate cancer cell proliferation.

According to the known inhibitory effect of LH-RH agonists and antagonists on the pituitary, it is anticipated that both classes of peptides would similarly cause inhibition of cell growth. Although such results were shown in several studies (5, 15), they were not reconfirmed by others (6) or by this study. In addition, the presence of a LH-RH antagonist binding site that is not recognizable by LH-RH agonists suggests that antagonists directly inhibit mammary tumor growth, not only by competing with LH-RH agonist binding sites, but also by other mechanisms mediated by an antagonist receptor.

Agonists and antagonists bind differently to pituitary and mammary tumor membranes. In the pituitary, only one binding site for LH-RH-like analogues is present (16). These receptors bind the peptides with high affinity. Such high-affinity receptors are present in mammary tumor cells (Fig. 3) and were found previously by us in 7,12-dimethylbenz-[*a*]anthracene-induced rat mammary tumor membranes (17). The binding of LH-RH agonists to these receptors was modulated by guanosine 5′-[γ-thio]triphosphate, suggesting that their signal transduction is mediated by GTP-binding proteins (13). However, in MCF-7 cells (Figs. 3 and 4), and in 7,12-dimethylbenz[*a*]anthracene-induced rat mammary tumors (13), additional low-affinity binding sites exist. The direct growth inhibition by antagonists, found in mammary tumor cells (Figs. 1 and 2), was achieved with a relatively high concentration (10^{-6}–10^{-5} M). These peptide levels correspond to the apparent binding constant of the low-affinity site in the mammary cancer cells.

Our results may provide some explanation for only a partial success achieved in the treatment of premenopausal breast cancer patients with LH-RH agonists. Santen *et al.* (18) reported recently on a 41% objective response in unselected patients and 51% in women with estrogen-receptor-positive tumors. The new generation LH-RH antagonists should be more suitable than the agonists for the treatment of breast cancer as they inhibit the growth of mammary tumors by two mechanisms: estrogen deprivation and a direct effect on the cells. The measurement of the low-affinity LH-RH antagonist binding in tumor biopsies may be important in predicting the response of breast cancer patients to antagonist therapy.

We are grateful to Drs. J. Sandow and W. Rechenberg (Hoechst, Frankfurt) for supplying buserelin and to Drs. R. Ghraf and Schroder (Ferring, Kiel, F.R.G.) for supplying [D-Trp^6]LH-RH. This work was supported by the Israeli Cancer Association and by the Israel Cancer Research Fund (New York).

1. Dickson, R. B. & Lippman, M. E. (1987) *Endocr. Rev.* **8,** 29–43.
2. Tolis, G., Ackman, D., Stellos, A., Mehta, A., Labrie, F., Fazekas, A. T. A., Comaru-Schally, A. M. & Schally, A. V. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 1658–1662.
3. Klijn, J. G. M. (1984) *Med. Oncol. Tumor Pharmacother.* **1,** 123–128.
4. Manni, A., Santen, R., Harvey, H., Lipton, A. & Max, D. (1986) *Endocr. Rev.* **7,** 89–94.
5. Miller, W. R., Scott, W. M., Morris, R., Fraser, H. M. & Sharpe, R. M. (1985) *Nature (London)* **313,** 231–233.
6. Eidne, K. A., Flanagan, C. A., Harris, N. S. & Millar, R. P. (1987) *J. Clin. Endocrinol. Metab.* **64,** 425–432.
7. Sharoni, Y., Bosin, E., Miinster, A., Levy, J. & Schally, A. V. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1648–1651.
8. Bajusz, S., Kovacs, M., Gazdag, M., Bokser, L., Karashima, T., Czernus, V. J., Janaky, T., Guoth, J. & Schally, A. V. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1637–1641.
9. Schally, A. V., Redding, T. W., Cai, R. Z., Paz, J. I., Ben-David, M. & Comaru-Schally, A. M. (1987) in *International Symposium on Hormonal Manipulation of Cancer: Peptides, Growth Factors and New (Anti) Steroidal Agents*, ed. Klijn, J. G. M. (Raven, New York), pp. 431–440.
10. Vignon, F., Bouton, M.-M. & Rochefort, H. (1987) *Biochem. Biophys. Res. Commun.* **146,** 1502–1508.
11. Carmaichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D. & Mitchell, J. B. (1987) *Cancer Res.* **47,** 936–942.
12. Fekete, M., Wittliff, J. L. & Schally, A. V. (1989) *J. Clin. Lab. Anal.* **3,** 137–147.
13. Segal-Abramson, T., Giat, J., Levy, J. & Sharoni, Y. (1992) *Mol. Cell. Endocrinol.*, in press.
14. Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107,** 220–239.
15. Bakker, G. H., Setyono-Han, B., Henkelman, M. S., de Jong, F. H., Lamberts, S. W. J., van der Schoot, P. & Klijn, J. G. M. (1987) *Cancer Treat. Rep.* **71,** 1021–1027.
16. Hazum, E. (1981) *Mol. Cell. Endocrinol.* **23,** 275–281.
17. Sharoni, Y., Segal-Abramson, T., Giat, Y., Kitroser, H., Bosin, E., Miinster, A., Feldman, B., Schally, A. V. & Levy, J. (1992) in *The Current Status of GnRH Analogues*, eds. Lunenfeld, B. & Insler, V. (Parthenon Publ. Group, Lancs, U.K.), in press.
18. Santen, R. J., Manni, A., Harvey, H. & Redmon, C. (1990) *Endocr. Rev.* **11,** 221–261.

Downloaded by guest on February 18, 2022